

# RESONANCE RAMAN STUDY OF THE PRIMARY PHOTOCHEMISTRY OF VISUAL PIGMENTS

## Hypsorhodopsin

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**ABSTRACT** We report here the first resonance Raman results of octopus hypsorhodopsin, a species formed photochemically at very low temperatures from visual pigments. A pump-probe technique was used to obtain Raman spectra from samples at 12°K whose photostationary state mixtures were either hypsorhodopsin rich or hypsorhodopsin poor. The data strongly suggest that the Schiff-base linkage between the chromophore of hypsorhodopsin and apoprotein is protonated. Further, the results suggest that hypsorhodopsin's chromophore is in some torsionally distorted conformation, possibly having torsional departures from an all-*trans* isomeric form.

## INTRODUCTION

An important question in the light energy transduction process in photoreceptors is the identity and structure of the primary photochemical species and the pathway that produces it from rhodopsin (1). In this step light energy is converted to chemical energy, which is eventually used in neural stimulation (2, 3). It is usually assumed that bathorhodopsin, whose absorption maximum lies to the red of rhodopsin, is the first photoproduct. However, a blue absorbing pigment, hypsorhodopsin, has been found in photostationary state mixtures of bovine and some other rhodopsins at liquid helium temperatures (4–7). It is unclear what role hypsorhodopsin plays in the primary photochemistry of rhodopsin, and/or whether its role is the same for all rhodopsins (7). Our recent low-temperature absorption results show that octopus rhodopsin has a hypso intermediate that is easily formed on irradiation, but that the batho-product appears before significant amounts of the hypso-product accumulate (7).

We report here the first resonance Raman measurements of octopus (*Paroctopus defleini*) rhodopsin at very low temperatures (12°K) to investigate structural properties of the chromophore of hypsorhodopsin. Of particular interest is the state of protonation of the chromophore-apoprotein Schiff-base linkage and the isomeric configuration of the chromophore. Our data strongly suggest that

the Schiff-base linkage of the chromophore of hypsorhodopsin is protonated. This result is perhaps surprising in view of hypsorhodopsin's blue-shifted absorption maximum, although certainly not outside theoretical feasibility.

## METHODS

Octopus (Mizudako, *Paroctopus defleini*) microvillar membranes were prepared as previously described (8). The composition of irradiated samples at liquid helium temperatures was determined by low-temperature absorption spectroscopy (7). For these measurements, rhodopsin was extracted from the membranes with a 2% digitonin solution, and glycerol was added to the preparation to give a final concentration of 75%.

A double wavelength pump-probe technique was used to record the resonance Raman spectra. The probe wavelength at 457.9 nm from an argon ion laser (model 165; Spectra-Physics Inc., Mountain View, CA) was used to stimulate the Raman scattering. Simultaneous application of a 530.9-nm pump laser beam, 15 times more intense than the probe power, from a krypton ion laser (model 52B; Coherent Radiation, Palo Alto, CA) then modified the photostationary state composition. Sample temperature was maintained at 12°K by a super-varitemp helium Dewar flask (Janis Research Co., Inc., Stoneham, MA). The Raman spectrometer, Dewar system, and procedures using the pump-probe technique have been described in detail elsewhere (9). The labeled bands' positions are accurate to within  $\pm 3$  cm<sup>-1</sup>.

Significant sample fluorescence background was observed. This was removed using the following procedure. In all runs, the background was represented by a straight line function and computer subtracted from the data. For experiments on protonated samples (see below), the background intensity at 800 cm<sup>-1</sup> was double the intensity of the dominant ethylenic

Raman peak (at  $1,534\text{ cm}^{-1}$ ) and five times at  $1,700\text{ cm}^{-1}$ . The background intensity for the deuterated sample data was about four times more intense than in the protonated data, although the low and high wave-number intensity ratio was essentially the same. The deuterated data thus contain considerably more noise. Separate scans on the deuterated samples were taken in the important  $1,500\text{--}1,700\text{ cm}^{-1}$  region, and the data were appropriately scaled and digitally pooled with that taken in the  $800\text{--}1,500\text{ cm}^{-1}$  region. This procedure increases the effective time constant and increases the signal-to-noise ratio in this range.

## RESULTS AND DISCUSSION

Fig. 1 shows two Raman spectra taken with a probe laser wavelength of  $457.9\text{ nm}$  from samples at  $12^\circ\text{K}$ . Fig. 1 *a* is the Raman spectrum with an additional coincident pump beam at  $530.9\text{ nm}$ , while Fig. 1 *b* is without the  $530.9\text{-nm}$  pump beam. Fig. 2 arises from identical experimental conditions except that the samples have been suspended in  $\text{D}_2\text{O}$ . The absorption measurements show that  $460\text{-nm}$  irradiation of samples at  $12^\circ\text{K}$  results in a photostationary state containing rhodopsin and isorhodopsin, bathorhodopsin, and hypsorhodopsin having relative concentrations of 60:35:5, respectively, while  $530\text{-nm}$  irradiation results in a 42:5:53 mixture. Thus, the spectrum of Fig. 1 *a* arises from samples that are hypsorhodopsin-rich, while that of Fig. 1 *b* is from hypsorhodopsin-poor samples.

The large relative increase in hypsorhodopsin concentration (shift in values, Fig. 1 *b* to *a*) is matched by a large increase in intensity of the  $1,565\text{ cm}^{-1}$  band. Bands in this region arise from ethylenic  $\text{—C=C—}$  stretching motions of the polyene moiety of the retinal chromophore. An empirical inverse linear relationship of chromophore absorption maximum vs. the frequency of the ethylenic Raman band has generally been observed for visual pig-

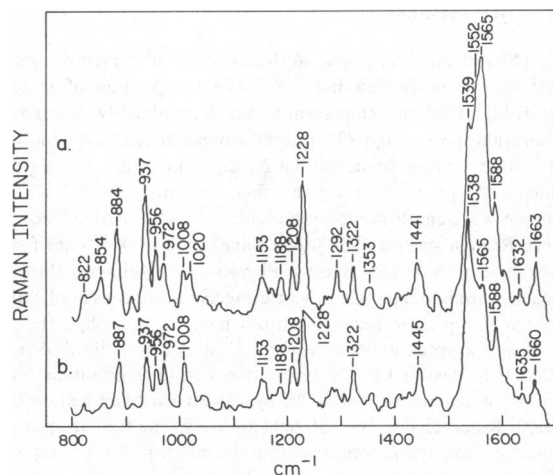


FIGURE 1 Resonance Raman spectra of normal (protonated) octopus rhodopsin and its photoproducts at  $12^\circ\text{K}$ . Spectral resolution is  $8\text{ cm}^{-1}$  and the data were smoothed using a five point Savitzky-Golay procedure (18). (a) Probe laser wavelength  $457.9\text{ nm}$ , and pumped with  $530.9\text{ nm}$  light. (b)  $457.9\text{ nm}$  probe beam only. The power ratios of pump/probe was 15:1.

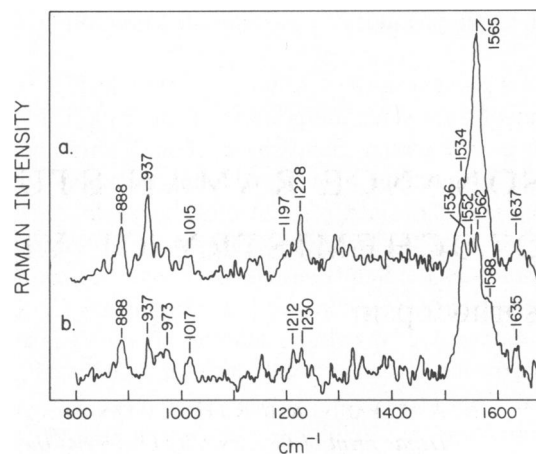


FIGURE 2 Resonance Raman spectra of deuterated octopus rhodopsin and its photoproducts at  $12^\circ\text{K}$ . (a) Probe laser wave length  $457.9\text{ nm}$  and pumped with  $530.9\text{ nm}$  light. (b)  $457.9\text{ nm}$  probe beam only. Except for deuteration, conditions are the same as in Fig. 1.

ments and other retinal systems (10). A  $1,565\text{ cm}^{-1}$  ethylenic band would correspond to an absorption maximum of  $\sim 440\text{ nm}$ , very close to the observed absorption maximum of hypsorhodopsin. We therefore assign the  $1,565\text{ cm}^{-1}$  band to hypsorhodopsin. In the same way, the  $1,538\text{ cm}^{-1}$  band is assigned to octopus bathorhodopsin and the  $1,552\text{ cm}^{-1}$  band to rhodopsin. The ethylenic band of isorhodopsin would lie near  $1,558\text{ cm}^{-1}$  and is apparently not resolved.

Reasonable band assignments to hypsorhodopsin can be made by contrasting the results of Fig. 2 with those of Fig. 1 and correlating the band intensity changes with changes in hypsorhodopsin concentration. Figs. 1 and 2 clearly show a dramatic increase in the amount of hypsorhodopsin in photostationary state mixtures in going from protonated to deuterated samples. The intensity of the hypsorhodopsin  $1,565\text{ cm}^{-1}$  marker band is at least five times more intense than any other ethylenic band in Fig. 2 *a*, a situation not true of the protonated spectrum (Fig. 1). This would indicate that the  $530.9\text{ nm}$  photostationary state of deuterated samples contains  $\sim 70\text{--}90\%$  hypsorhodopsin as opposed to  $\sim 50\%$  for protonated samples. Since hypsorhodopsin so dominates the deuterated spectrum, it seems safe to assign the dominant  $888, 937, 1,015, 1,197,$  and  $1,228\text{ cm}^{-1}$  bands to hypsorhodopsin. The assignment of the  $888, 937,$  and  $1,228\text{ cm}^{-1}$  to hypsorhodopsin is strengthened by the observation that the intensity of these bands in the protonated samples decreases in changing the sample composition from hypsorhodopsin rich (Fig. 1 *a*) to hypsorhodopsin poor (Fig. 1 *b*). The two small intensity bands at  $1,020$  and  $1,292\text{ cm}^{-1}$  observed in the hypsorhodopsin-rich spectrum of Fig. 1 *a* also undergo an intensity decrease in the hypsorhodopsin poor spectrum of Fig. 1 *b*. It is thus reasonable to tentatively assign these bands to hypsorhodopsin as well. Unfortunately, the relatively larger noise present in the data of Fig. 2 *a* in the  $800\text{--}1,500\text{ cm}^{-1}$

spectral range precludes a clear identification of these two bands.

The assignment of the  $\sim 1,660\text{ cm}^{-1}$  band in Fig. 1 is of great importance since the position of this band is indicative of a protonated Schiff-base retinal chromophore ( $\text{C}=\text{NH}^+$  stretching mode) (11); unprotonated Schiff-base retinals show a  $\text{C}=\text{N}$  stretching mode at lower frequencies  $\sim 1,630\text{--}1,620\text{ cm}^{-1}$ . Suspension of samples in  $\text{D}_2\text{O}$  deuterates the Schiff base by exchange and results in protonated Schiff-base bands at lower frequency. Comparing the data of Fig. 1 with the deuterated data of Fig. 2, a downward shift from  $\sim 1,660\text{ cm}^{-1}$  to  $1,637\text{ cm}^{-1}$  is observed. That rhodopsin, isorhodopsin, and bathorhodopsin form protonated Schiff-base chromophore-apoprotein linkages is expected because their absorption maxima are considerably red-shifted from model unprotonated Schiff bases and, also, because these species have been demonstrated to be protonated in the bovine visual pigment systems (11).

The data of Fig. 1 also strongly imply that hypsorhodopsin contains a protonated Schiff-base chromophore. Careful examination of the data shows that the intensity of the  $1,663\text{ cm}^{-1}$  band in the hypsorhodopsin-rich spectrum of Fig. 1 *a* is at least as intense as the hypsorhodopsin-poor (Fig. 1 *b*)  $1,660\text{ cm}^{-1}$  band. In fact, the hypsorhodopsin-rich  $1,663\text{ cm}^{-1}$  band is apparently 30% larger than the hypsorhodopsin-poor  $1,660\text{ cm}^{-1}$  band. On the other hand, the noise in this spectral range is about  $\pm 10\%$  of the  $1,663\text{ cm}^{-1}$  intensity, and this precludes a quantitative statement that the protonated Schiff-base band increases upon hypsorhodopsin formation. However, judging from the hypsorhodopsin  $1,565\text{ cm}^{-1}$  marker band, hypsorhodopsin contributes  $\sim 50\%$  of the Raman scattered light in Fig. 1 *a*. If hypsorhodopsin contained an unprotonated Schiff base and assuming that the Raman intensity ratios of the protonated Schiff-base mode to ethylenic mode are nearly the same for rhodopsin, isorhodopsin, and bathorhodopsin (which is true for the bovine species [1, 11]), we would have expected to observe an increase of  $\sim 50\%$  in the  $\sim 1,660\text{ cm}^{-1}$  band in going from hypsorhodopsin-rich to hypsorhodopsin-poor conditions. (Note that Schiff-base retinal chromophores have no other normal modes in the  $\sim 1,660\text{ cm}^{-1}$  region.) This is not observed; certainly no increase (and perhaps a small decrease) of the  $1,660$  band is seen. This suggests that hypsorhodopsin has a  $1,660\text{ cm}^{-1}$  band and thus is a protonated Schiff base. There is a caveat, however. The pump laser beam of  $530.9\text{ nm}$  not only enriches the hypsorhodopsin in the photostationary state but also isorhodopsin, though, to a much lesser extent. An unusually high Raman intensity of the octopus isorhodopsin's protonated Schiff-base mode could result in the observed Raman intensity patterns without regard to hypsorhodopsin's contribution.

The blue-shifted absorption spectrum of hypsorhodopsin is perhaps surprising in view of our inference that this species forms a protonated Schiff-base linkage. There are,

however, a number of plausible explanations. Because the absorption maximum of hypsorhodopsin is close to that of a protonated Schiff base of retinal in solution, hypsorhodopsin may arise from a similar situation; that is, in hypsorhodopsin the chromophore may have moved far away from the "second negative charge" postulated (12, 13) to red-shift the absorption maximum of the *in situ* retinal protonated Schiff base relative to the solution value. Another possibility for the blue shift is extensive single-bond twisting (1). A third is that upon formation of hypsorhodopsin, one of the negatively charged groups around the chromophore that control its color becomes protonated. At present we have no way of distinguishing between these possibilities.

Our results do suggest that hypsorhodopsin's chromophore is in some torsionally distorted conformation, possibly having torsional departures from an all-*trans* isomeric form. The so-called fingerprint spectral region, from  $\sim 1,100\text{--}1,400\text{ cm}^{-1}$ , is quite sensitive to the retinal isomeric configuration and pronounced spectral pattern changes are observed from one isomer to another. The lower frequency bands, below  $\sim 1,000\text{ cm}^{-1}$ , have been assigned to arise from vinyl hydrogen out of plane bending modes by Mathies, Lugtenburg, and their collaborators (14). Significant Raman intensity from these modes can be explained by twists about retinal chain single bonds. Thus the observation of strong bands at  $888$  and  $937\text{ cm}^{-1}$  that we have assigned to hypsorhodopsin suggest torsional departures from planarity. Hypsorhodopsin's fingerprint spectral characteristics, essentially one strong dominating band at  $1,227\text{ cm}^{-1}$ , are found in spectra of both the all-*trans* protonated Schiff base of retinal in solution and cattle bathorhodopsin (1, 15, 16), a pigment thought to contain a torsionally distorted all-*trans* chromophore. The low frequency pattern of bands in the octopus hypsorhodopsin spectrum is not the same as cattle bathorhodopsin (which has strong bands at  $856$ ,  $877$ , and  $920\text{ cm}^{-1}$ ), suggesting that the torsional distortions from planarity are different for the two pigments.

The result that the proportion of hypsorhodopsin in photostationary state mixtures is altered by sample deuteration is interesting, suggesting deuteration affects yields of the photochemistry. Such deuteration effects are not seen for the photochemistry at  $77^\circ\text{K}$  where only mixtures of rhodopsin, isorhodopsin, and bathorhodopsin are observed (17). The deuteration effect on quantum yield must be associated with the exchangeable Schiff-base proton or exchangeable protons of the apoprotein amino acid residues, but at present we cannot distinguish between these two possibilities.

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